REMARKS

Upon entry of this amendment, claims 14, 46, 48, and 49 are pending in the instant application. Claims 45 and 50-54 have been withdrawn from consideration by the Examiner as being drawn to non-elected subject matter. Applicants have cancelled claims 45, 47, and 50-54, without prejudice or disclaimer. Applicant reserves the right to prosecute the cancelled subject matter, as well as the originally presented claims, in continuing applications. No new matter has been added.

FORMAL MATTERS

Applicant acknowledges that the amendment to the specification regarding the priority claim has been entered.

Applicant notes with appreciation that the objection to claim 14 for being drawn to non-elected species has been withdrawn.

Additionally, Applicant notes with appreciation that the rejections of claim 14 under 35 U.S.C. § 112, first paragraph for lack of adequate written description and under 35 U.S.C. § 112, second paragraph have been withdrawn.

Finally, Applicant notes that the Information Disclosure Statement filed on January 30, 2004 has been considered.

CLAIM REJECTION UNDER 35 U.S.C. § 112, SECOND PARAGRAPH

Claims 48 and 49 stand rejected under 35 U.S.C. § 112, second paragraph as being indefinite. In particular, the Examiner states that there is insufficient antecedent basis for the

terms "the cells." As amended, these claims now recite the terms "the pancreatic B-cell," which find antecedent basis in claim 14. This objection may now be withdrawn.

CLAIM REJECTIONS UNDER 35 U.S.C. § 102

Smith

Claims 14 and 46-48 stand rejected under 35 U.S.C. § 102(b) as being anticipated by WO 93/18759 ("Smith"). The Examiner states that Smith provides methods for targeting DNA across cellular or nuclear membranes using peptide ligands that contain Applicant's claimed sequence. Relying on the teaching that cells expressing the insulin-like growth factor I (IGF-1) receptor may be amenable to such targeting strategies, the Examiner concludes that Smith discloses the claimed invention. Applicant traverses this rejection.

As amended, claim 14, from which claims 46, 48, and 49 depend, is directed to a method of translocating a transporter peptide having the amino acid sequence of SEQ ID NO:1 (RRTK) into a *pancreatic B-cell.* Regarding the issue of novelty, the case law states: "[a] claim is anticipated *only if* each and every element as set forth in the claim is found, either *expressly* or *inherently* described, in a single prior art reference." (emphasis added) *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). Applying this standard to the present case, Applicant asserts that Smith does not disclose every limitation of the claimed method because Smith fails to teach or suggest targeting molecules into pancreatic B cells. In addition, Smith further fails to teach Applicant's claimed sequence (RRTK). In contrast, Smith only discloses peptides that contain at least 13 amino acids.

Although the Examiner states that Smith, in teaching methods to target the IGF-1 receptor, inherently discloses the present invention, Applicant disagrees and submits that the

IGF-1 receptor is <u>not</u> expressed exclusively by pancreatic cells. Rather, the IGF-1 receptor is expressed by a multitude of cell types including, for example, tumor cells, muscle cells, neurons, and osteoclasts (see, for example, page 17, lines 8-9 of the specification). As evidence of this assertion, Applicant hereby submits Exhibits A-C. Exhibit A (Baserga, Receptor 2:261-266, 1992) states, for example:

> The IGF-1 receptor is expressed in many cell types, and its activation by its ligands is a required step for the proliferation of many cells in vivo and in vitro...It is also well established that most cells in culture require IGF-1 for growth: these include fibroblasts, keratinocytes, hemopoietic cells, smooth muscle cells, mammary epithelial cells, osteoblasts, chondrocytes, and many others...In fact, only a few cell types seem to be devoid of IGF-1 receptors, for instance hepatocytes and certain B cell lines.

Exhibit B (Werner et al., Cell. Mol. Life Sci. 57: 932-942, 2000) further states "the IGF-1-R is highly overexpressed by most tumors and cancer cell lines," while Exhibit C (Van Wyk et al., J. Clin. Endo. Met. 84: 4349-4354, 2004) states "[b]oth IGF-I and IGF-II produce their biological effects through types I receptors that are homologous with the insulin receptor...Type I receptors are present in most, if not all, tissues." Accordingly, a teaching that a molecule may be targeted to cells expressing the IGF-1 receptor does not necessarily or automatically imply the use of pancreatic B-cells and <u>Smith</u>, as a result, does not inherently teach the claimed method. In fact, the case law states in this regard:

> To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is *necessarily* present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.' " (Emphasis added) *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999)

Thus, because <u>Smith</u> does not expressly or inherently teach every limitation of the claimed method, this § 102 rejection should be withdrawn.

Woo

Claims 14 and 46-48 further stand rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent No. 5,994,109 ("Woo"). The Examiner states that Woo teaches methods for translocating nucleic acids through nucleic membrane employing peptides that contain the claimed peptide. Because Woo teaches that these methods are useful for cells expressing IGF-1 receptor and because pancreatic cells express this receptor, the Examiner contends that Woo discloses the present invention. Applicant traverses this rejection.

Applicant submits that Woo, like Smith, does not expressly teach or suggest transporting molecules across the membranes of pancreatic B-cells, as required by the instant claims. Nor does Woo inherently provide such a teaching. As is stated above, a teaching that a molecule may be targeted to cells expressing the IGF-1 receptor does not inherently teach that pancreatic B-cells may be employed. Woo further fails to teach or suggest the claimed sequence since the peptide containing Woo's SEQ ID NO: 1 (peptide 8) has an additional 8 amino acids. Moreover, Woo teaches that this peptide functions as a nuclear localization sequence, therefore binding a nuclear receptor rather than a cell surface receptor (see column 8, lines 10-24 in Woo). Thus, because Woo does not expressly or inherently teach every limitation of the claimed method, this § 102 rejection should be withdrawn.

Robbins

Claims 14 and 46-49 stand rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. patent application publication 2003/0104622 ("Robbins") for teaching methods to deliver molecules into pancreatic B-cells using peptides that contain SEQ ID NO: 1. Applicant traverses this rejection.

As stated by the Examiner, Robbins teaches methods for delivering molecules into cells using peptide ligands that contain specific amino acid sequences. Although Robbins teaches that pancreatic B-cells are amenable to these methods, Robbins, however, fails to teach that molecules may be delivered to pancreatic B-cells by means of a transporter peptide of SEQ ID NO:1, as is required by the claims. Thus, because Robbins fails to teach every limitation of the claim, Applicant respectfully requests that this rejection be withdrawn.

In view of the foregoing, Applicant respectfully requests that the claim rejections under 35 U.S.C. § 102 be withdrawn.

CLAIM REJECTIONS UNDER 35 U.S.C. § 103(a)

Claims 14 and 46-49 stand rejected under 35 U.S.C. § 103(a) as being obvious in view of Smith, Robbins, or alternatively, Woo. In applying this rejection, the Examiner states that it would have been obvious to formulate pharmaceutical compositions to deliver molecules into pancreatic B-cells using transporter peptides that contain SEQ ID NO:1 as taught by Smith, Robbins, or Woo. Applicant traverses this rejection.

As discussed above, the claims of the present invention are directed to methods of translocating a transporter peptide of SEQ ID NO: 1 into pancreatic B-cells and are based on Applicant's discovery that the amino acid sequence RRTK (SEQ ID NO: 1) can specifically and efficiently target pancreatic B-cells (see page 24, lines 10-11 of the specification). As a preliminary matter, Applicant points out to the Examiner that the peptide sequences disclosed by Smith, Robbins, and Woo, while containing the sequence of SEQ ID NO: 1, are all significantly larger than the claimed peptide sequence, which only contains four amino acids. In particular, the Smith, Robbins, and Woo peptides contain at least an additional 8, 10, 8 amino acids,

respectively. Applicant submits that knowledge of these peptide sequences does not teach or suggest the existence or function of the claimed peptide sequence. Accordingly, one skilled in the art reading Smith, Robbins, and Woo, alone or in combination, would not recognize, much less be motivated, to translocate the claimed transporter peptide (SEQ ID NO: 1) into a pancreatic B-cell or to formulate any pharmaceutical compositions for the same purpose.

Turning now to the first cited reference, Smith never teaches the use of pancreatic B-cells, as is claimed. Smith only goes so far as to suggest that nucleic acids attached to a peptide may be delivered to cells that express the IGF-1 receptor. As discussed above, because the IGF-1 receptor is expressed by a variety of cells, this teaching would hardly motivate those of skill in the art seeking to practice the claimed invention to use pancreatic B cells. Applicant further notes that because Smith discloses over 20 peptide sequences that may be useful for the intracellular delivery of molecules, those of skill in the art would not have known to chose a peptide sequence having the sequence of SEQ ID NO:1, let alone use such a peptide for the delivery of molecules to pancreatic B cells.

Likewise, one skilled in the art reading Robbins would not be motivated to practice the claimed invention. Robbins discloses over 90 peptides (see Tables 1-6 in Robbins) useful for the intracellular delivery of molecules, but never teaches or suggests the use of a peptide having the claimed sequence for translocating into a pancreatic B-cell. While Robbins teaches methods to deliver molecules into a variety of cells (including pancreatic B-cells) using any of the enumerated peptides, there is no teaching or suggestion to chose a peptide of SEQ ID NO:1 (RRTK) for translocation into a pancreatic B-cell. None of the peptide sequences disclosed by Robbins as being useful for translocation of pancreatic B-cells (SEQ ID NOs: 1-6, 59, and 86 in Robbins) contain SEQ ID NO: 1. Thus, if anything, one skilled in the art reading Robbins would

have been motivated to use one of these sequences rather than the claimed sequence to deliver molecules to pancreatic B-cells.

Similarly to Smith, the third cited reference, Woo, fails to teach delivery of molecules to pancreatic B-cells. Rather, Woo teaches the delivery of molecules into cells expressing the IGF-1 receptor. In fact, Woo suggests targeting the IGF-1 receptor in muscle cells and osteoblasts and is silent on the targeting of pancreatic B-cells (see column 88, line 35 to column 90, line 24 in Woo). Moreover, Woo teaches that the delivery of molecules into IGF-1 receptor-expressing cells may be achieved using a particular peptide sequence that does *not* contain the claimed sequence. Rather, Woo teaches that the peptide containing the claimed sequence is useful for nuclear localization. Thus, one skilled in the art reading Woo would not have been motivated to deliver molecules to pancreatic B-cells or to use the claimed sequence to do so.

In view of the foregoing, Applicant submits that one skilled in the art reading Smith, Robbins, or Woo, alone or in combination, would not have been motivated to deliver molecules into pancreatic B cells by means of the translocation peptide of SEQ ID NO: 1 or to formulate pharmaceutical compositions for the same purpose. Accordingly, the § 103(a) rejection should be withdrawn.

CONCLUSION

Applicant respectfully submits that the claims are in condition for allowance. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

Respectfully submitted,

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Exhibit A

Receptor

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The Double Life of the IGF-1 Receptor

Minireview

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Contents

Abstract

The IGF-1 receptor is expressed in many cell types, and its activation by its ligands is a required step for the proliferation of many cells in vivo and in vitro. In most cells in culture, requiring more than one growth factor for growth, the IGF-1 receptor can be found in one of two different modes: in the first mode, although it is autophosphorylated by its ligands and induces the expression of specific genes, it does not transmit a mitogenic signal. In the alternative mode, i.e., after priming with an unrelated growth factor, the IGF-1 receptor responds to its ligands with a mitogenic stimulus. This review examines briefly the possible alternatives to explain this different behavior, which is crucial to our understanding of the control of cellular proliferation.

Index Entries: IGF-1; IGF-1 receptor; cell cycle; growth factor; cellular proliferation.

Introduction

The activation of the IGF-1 receptor by its ligands (IGF-1, IGF-II, or insulin at supraphysiological concentrations) plays a major role in the control of cellular proliferation in mammalian cells (for reviews, *see* LeRoith et al., 1991; Lowe, 1991). Its importance in embryonal development is now established: Efstratiadis and coworkers (DeChiara et al., 1990) had shown that the targeted disruption of the IGF-II gene results in a 30% decrease in murine embryo growth. At a recent meeting, Efstratiadis showed evidence that the targeted disruption of both the IGF-II and the IGF-1 receptor genes leads to 30% dwarfism, i.e., the homozygous embryos are 30% the weight of wild type embryos. These fascinating studies therefore indicate that 70% of murine embryonal growth is under the control of the IGF-1 receptor (IGF-II exerts its growth-promoting activity through the IGF-1 receptor); at the same time, they tell us that 30% of embryonal growth is outside the jurisdiction of the IGF-1 receptor, launching a quest for the receptor and ligand that act independently of the IGF-1 receptor.

It is also well established that most cells in culture require IGF-1 for growth: these include fibroblasts, keratinocytes, hemopoietic cells, smooth muscle cells, mammary epithelial cells, osteoblasts, chondrocytes, and many others (for a review, *see* Lowe, 1991). In fact, only a few cell types seem to be devoid of IGF-1 receptors, for instance hepatocytes and certain B cell lines. But although most people will agree that the activation of the IGF-1 receptor by its ligands plays an important role in mammalian cell proliferation, there is a puzzling feature in its bioeffects, whose solution may very well hold the key to our understanding of cell cycle controls. An analysis of this puzzle is the object of this minireview.

The Facts

We shall take fibroblasts, especially 3T3 cells, as an illustration, but one should remember that the same situation applies to other cell types. 3T3 cells, as is well known, require more than one growth factor for optimal growth (Scher et al., 1979), usually PDGF and IGF-1. Either of the two growth factors, by themselves, are incapable of stimulating growth. Thus, addition of IGF-1 alone to 3T3 cells (Pietrzkowski et al., 1992a) or to WI-38 human diploid fibroblasts (Cristofalo et al., 1989) fails to elicit a mitogenic response. But if the cells are previously or simultaneously incubated with PDGF (or EGF, in the case of WI-38 cells), then the addition of IGF-1 results in the optimal stimulation of cellular proliferation. There would be no puzzle if quiescent 3T3 cells had no receptors for IGF-1, and if these receptors were to appear only after stimulation with PDGF.

The puzzle is that IGF-1 receptors are present in growth-arrested cells (Clemmons et al., 1986), they are autophosphorylated by the appropriate ligands (Pietrzkowski et al., 1992b) and transmit a signal resulting in growth in size of the cells (Zetterberg et al., 1984), and the expression of specific genes, such as ribosomal RNA genes (Surmacz et al., 1987), c-fos (Damante et al., 1988), c-jun (Chiou and Chang, 1992), and a transcription factor for thyroglobulin (Santisteban et al., 1992). But 3T3 cells stimulated by IGF-1 alone do not synthesize DNA nor divide.

The Problem

Clearly, the IGF-1 receptor is leading a double life. By itself, it is active but not mitogenic; with the cooperation of other growth factors, it becomes mitogenic. What changes the IGF-1 receptor from its nonmitogenic mode to its mitogenic mode is crucial to the control of cell proliferation, not only because its activation is required for growth by many types of cells, but also because the cell cycle clock starts with the activation of the receptor in its mitogenic mode. Thus, if 3T3 or WI-38 cells are primed with PDGF or EGF, a delay in the addition of IGF-1 results in a proportional delay in the entry of cells into S phase (Cristofalo et al., 1989; Yoshinouchi and Baserga,

1993). The other growth factors are necessary, but cell cycle progression is timed by the IGF-1 receptor.

Possible Explanations

The number of possible explanations for the conversion of the IGF-1 receptor from its non-mitogenic to its mitogenic mode is, of course, almost limitless, but we can group them into three large categories:

1. The receptors for IGF-1 and the other growth factors act through completely independent pathways, both of which must be completed for the cells to enter S phase and divide;
2. The first growth factor (PDGF or EGF or IL-2, or others) induces an increase in the number of IGF-1 receptors, in other words, a quantitative change that makes the activated IGF-1 receptor mitogenic; and
3. The other growth factors induce a qualitative modification of the IGF-1 receptor, where the term qualitative should be intended in a broad sense as any change in the receptor itself, its substrates, or its signal transducing pathways.

Pros and Cons

Independent Pathways

By independent pathways, we mean cell cycle pathways, for instance, each growth factor could induce the expression of two different sets of genes, each individual set being necessary but not sufficient for cell proliferation. In favor of this hypothesis is the fact that PDGF induces the mRNAs for early growth regulated genes (Lau and Nathans, 1985; Almendral et al., 1988), most of which are different from IGF-1 induced mRNAs (Zumstein and Stiles, 1987). Late growth-regulated genes, as for instance DNA synthesis genes, like DNA polymerase alpha, PCNA, thymidine kinase, and so on, are usually not induced by either PDGF alone or IGF-1 alone, but require the intervention of both growth factors. This suggests another way by which independent pathways could be operating, which is supported by some tantalizing results indicating that the transcription of certain DNA synthesis genes begins very early after growth stimulation, but the mRNAs do not become detectable until much later (Gudas et al., 1988; Lipson and Baserga, 1989). If these findings are confirmed, then the function of the IGF1 receptor is to activate the processing of the pre-mRNAs for the DNA synthesizing genes, which would be in agreement with data from Zumstein and Stiles (1987) and Koniecki et al., (1991). Since the products of the DNA synthesis genes are required for cell proliferation, the IGF-1 receptor by itself could not be mitogenic, simply because the pre-mRNAs would not be available for processing.

Quantitative Change

It has been known for a number of years that certain growth factors, like PDGF and EGF, induce an increase in the number of IGF-1 binding sites (Clemmons and Van Wyk, 1981; Clemmons and Shaw, 1983). Recently, in our laboratory, we have shown that Interleukin-2 induces a spectacular increase in the levels of IGF-1 receptor RNA in T lymphocytes (Reiss et al., 1992), and confirmed in human diploid fibroblasts that PDGF produces a 2–3-fold increase in the number of IGF-1 receptors that can be autophosphorylated (Sell et al., submitted). The simplest interpretation of these results is that the number of IGF-1 receptors is critical, and that, by increasing the number of receptors, the other growth factors render the cells capable of responding with mitosis to the IGF-1 stimulus. This interpretation is supported by several recent observations that cells constitutively overexpressing the IGF-1 receptor grow in serum-free medium, with IGF-1 as the only exogenously added growth factor (McCubrey et al., 1991; Pietrzkowski et al., 1992a).

Against this hypothesis are essentially two observations:

1. There are a number of cell lines that display high levels of IGF-1 receptors, which respond to IGF-1, not with proliferation, but with differentiation (Ota et al., 1989); and
2. In cells overexpressing the IGF-1 receptor, mentioned above, IGF-1 induces the expression of certain genes, like c-myc, that are usually induced by PDGF (Surmacz et al., 1992).

In other words, it is possible that constitutive overexpression of the IGF-1 receptor may activate pathways that are not activated under physiological conditions. It should be possible, very soon, to establish whether a slight increase in the number of IGF-1 receptor is necessary for the mitogenic response. It will be more difficult to determine if it is not only necessary but also sufficient.

Qualitative Change

The meaning of qualitative, in this context, includes any modification in the receptor itself, its substrates, or the signal transducing pathway. One type of qualitative change has been described in the case of the PDGF beta receptor. Binding of PDGF to its receptor results in tyrosine phosphorylation of multiple proteins, including the receptor itself, which is phosphorylated at three tyrosine residues and binds to the SH2 domains of at least two proteins, a GTPase activating protein (GAP) and phosphatidyl-inositol 3-kinase (PI3-kinase). Fantl et al., (1992) showed that in the mouse PDGF-beta receptor, phosphorylation of tyrosine residues 708 and 719 are necessary for binding to the p85 subunit of PI3-kinase and for mitogenesis, whereas tyrosine 739 associates with GAP and can be replaced by another amino acid without affecting the mitogenic response to PDGF. Similar results were obtained by Kazlauskas et al., (1992) with the human PDGF receptor. This duality of substrates, one mitogenic and the other nonmitogenic, has been confirmed with another growth factor receptor. A point mutation in a FGF receptor abolished phosphatidyl turnover and calcium fluxes without affecting mitogenesis (Peters et al., 1992; Mohammadi et al., 1992).

Thus, an alternative explanation for the double life of the IGF-1 receptor would be that PDGF (or EGF, or IL-2, or an overexpressed IGF-1 receptor) mobilizes a specific IGF-1 receptor substrate that is not available in quiescent cells, not previously primed by another growth factor. In other words, the ability of the IGF-1 receptor to stimulate or not cell proliferation would depend on the cell context. An illustration of the importance of the cell context can be found in the report by Renshaw et al., (1992) that the v-abl tyrosine kinase can inhibit or stimulate growth and requires a permissive cellular context to manifest its mitogenic function. The concept of a cellular context could also explain the fact that activation of the IGF-1 receptor can, in some cases, induce, not cellular proliferation, but cellular differentiation, as mentioned above.

Conclusions

There are, unfortunately, no conclusions. Of all the growth factor receptors, the IGF-1 receptor is by far the least studied. We do not even know which tyrosines are autophosphorylated, which ones are necessary for its mitogenic effect, and the little we know about its possible substrates (like IRS-1 or the p85 subunit of PI3-kinase) are largely based on analogies with the insulin receptor, although a few studies with the IGF-1 receptor have been recently published (Yamamoto et al., 1992). Yet, these studies are of the utmost importance, because the cell cycle clock starts with the activation of the IGF-1 receptor in what we call its mitogenic mode (Cristofalo et al., 1989; Yoshinouchi and Baserga, 1993). To identify the mechanism that changes the IGF-1 receptor from a nonmitogenic to a mitogenic mode (whether it is in the receptor itself, its substrates or further downstream) is to identify the most important single step that controls the proliferation of cells.

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Exhibit B

CMLS, Cell. Mol. Life Sci. 57 (2000) 932–942
1420-682X/00/060932-11 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

New concepts in regulation and function of the insulin-like growth factors: implications for understanding normal growth and neoplasia

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Abstract. The insulin-like growth factors (IGFs) are a ubiquitous family of growth factors, binding proteins and receptors that are involved in normal growth and development. They are also implicated in numerous pathological states, including malignancy. IGF-II is a commonly expressed growth factor in many tumors and may enhance tumor growth, acting via the overexpressed IGF-I receptor, a cell-surface tyrosine kinase receptor. The IGF-I receptor may be overexpressed due to mutations in tumor suppression gene products such as p53 and WT-1 or growth factors such as bFGF and PDGF. Thus, this family of growth factors, especially the IGF-I receptor, may present an excellent target for new therapeutic agents in the treatment of cancer and other disorders of excessive cellular proliferation.

Key words. Insulin-like growth factors; IGF-I receptor; cancer; receptor signaling.

Introduction

Over the last several years, the insulin-like growth factor (IGF) field has witnessed an overwhelming influx of new information of both experimental and clinical nature (for a recent update see [1]). Some of these novel findings were quite unexpected, and they have compelled us to review and update a number of basic concepts that have prevailed for more than 30 years. The purpose of this essay is to review some of these new data and to evaluate their significance in the specific context of various physiological and pathological processes that involve the IGF system.

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The IGF family: old and new

The critical elements that regulate IGF function include ligands, receptors and IGF-binding proteins (IGFBPs). To date, this family comprises three ligands (insulin, IGF-I and IGF-II), three cell-surface receptors (the insulin, IGF-I and IGF-II/mannose-6-phosphate receptors) and at least six IGFBPs, which bind circulating IGFs and modulate their function. In addition to these 'classical' family members, which have been well characterized, more recent work has identified several other proteins as potential components of the IGF system. These 'nonclassical' members include two additional receptors (the insulin-receptor-related receptor (IRR) and the insulin-IGF-1 hybrid receptor), and a steadily

growing number of IGFBP-related proteins. In addition, the biological activities of the IGFs have been shown to be modulated by a group of IGFBP-proteases that cleave the binding proteins, thereby regulating the overall availability of these ligands. The number and specificity of the various proteases involved has yet to be elucidated.

Both IGF-I and IGF-II are produced primarily by the liver, which is the major source of endocrine IGFs. IGF-I biosynthesis is tightly correlated with circulating levels of growth hormone (GH). Consequently, IGF-I gene expression levels increase 10- to 100-fold between birth and adulthood [2]. In contrast, IGF-II messenger RNA (mRNA) levels in rodents are high during the fetal and perinatal periods and decline thereafter [3]. In humans, however, significant levels of circulating IGF-II can be detected in adults. The original somatomedin hypothesis was based on the findings that GH can stimulate sulfate and thymidine incorporation into cartilage and that these effects are mediated by a plasma-borne factor ('sulfation factor', later termed IGF-I) [4]. Subsequent studies demonstrated that multiple tissues are capable of synthesizing IGFs, during both the fetal and adult stages of development. These findings provided evidence that IGFs can act locally (i.e. autocrine/paracrine) in addition to their endocrine modes of action [3, 5]. Finally, it was recently reported that ablation of IGF-I production specifically in the liver has essentially no effect on the growth and development of mice [6]. The potential implications of this unexpected finding will be discussed below.

The second component of the IGF family is the set of cell-surface receptors. There is ample consensus today that much of the biological action of the IGFs on growth and differentiation is mediated by the IGF-I receptor (IGF-I-R). A detailed description of the cellular events associated with activation of this receptor will be presented in the next section. The insulin receptor (IR) is very similar to the IGF-I-R both in overall gene and protein organization and with respect to the intracellular components that mediate insulin signal transduction. Despite these striking similarities, the IR is significantly less potent than the IGF-I-R in inducing mitogenesis. However, if IGF-II is present in abundance, then the IR can contribute significantly to mitogenesis [7]. The IGF-II/mannose-6-phosphate receptor (IGF-II/Man-6-P) receptor is a bifunctional binding protein that binds both IGF-II and ligands that contain Man-6-P at distinct binding sites. Whereas it has been clearly established that the IGF-II/Man-6-P receptor functions in lysosomal enzyme trafficking and IGF-II degradation, its role in IGF signal transduction remains controversial [8, 9].

For a number of years it has been suggested that there are atypical insulin and IGF receptors that are capable of binding one or both ligands with relatively high affinity [10]. Receptor heterogeneity may result from primary structure variation, differential glycosylation, splicing events, hybrid formation, and additional gene products such as the IRR [11]. The IRR shares ~55% identity at the amino acid level with that of the IR and IGF-I-R [12]. The C-terminal domain downstream of the tyrosine kinase region of the IRR, however, is significantly shorter. The IRR does not bind significant levels of IGF-I, IGF-II, insulin, proinsulin or relaxin, and thus to date the IRR remains an orphan receptor with no identified ligand [13].

Among the various atypical receptors reported, attention has mostly been focused on the hybrid IR/IGF-I-R. It has now been clearly established that hybrid heterodimeric receptors comprising an IR α-β 'hemireceptor' and an IGF-I-R α-β hemireceptor form in cells. This has been demonstrated by several approaches, including sequential immunodepletion and immunoprecipitation experiments [14], immunoprecipitation of the IGF-I-R with a monoclonal antibody (αIR3) followed by microsequencing [15], and expression of full-length IGF-I-R and C-terminal truncations of the IR in HeLa cells [16]. IR/IGF-I-R hybrids seem to be widely expressed and, in certain tissues, even appear to be the most abundant form of receptor [17]. However, specific differences in signaling characteristics between the IGF-I-R and IR/IGF-I-R hybrids have not yet been established.

Finally, a virtual explosion of new information occurred in the field of the IGFBPs, the third component of the IGF system. This IGFBP superfamily includes, in addition to the classical members, IGFBP-1 through IGFBP-6, several other members which are now termed IGFBP-related proteins (IGFBP-rP)-1 through IGFBP-rP-5 [18, 19]. The IGFBP superfamily seems to have evolved from a common ancestor, with some of its current members showing high affinity for IGF, whereas others display low affinity. An important implication of this finding is that the IGFBP superfamily may influence cell growth in both IGF-dependent and IGF-independent fashion.

Signal transduction through the IGF-I receptor

The IGF-I-R is a member of the tyrosine kinase receptor family and is closely related to the insulin receptor. Both receptors form a subclass, because unlike other members of the family that are single transmembrane proteins, both the insulin and IGF-I-R exist as preformed dimers. Both receptors consist of two α and two β subunits joined by disulfide bonds [11, 20]. Ligand binding to the extracellular α subunits results in a conformational change that induces autophosphoryla-

tion of tyrosine residues within the β subunit, which is primarily intracellular. Autophosphorylation stimulates the receptor tyrosine kinase activity and leads to phosphorylation of other substrates. A number of various SH2 domain-containing proteins or 'docking proteins' bind to specific phosphotyrosine residues in the C-terminal portion of the β subunit. Some of these proteins also become phosphorylated by the receptor tyrosine kinase. The insulin receptor substrate (IRS) family of proteins (IRS-1 through IRS-4) and Shc are the best-characterized docking proteins (fig. 1). These proteins can then bind SH2-containing proteins in a manner dependent on the specific phosphotyrosine motif (Y-X-X-X) involved [21, 22]. These SH2-containing proteins include GRB-2, which together with mSOS can activate Ras, SH-PTP2 (a tyrosine phosphatase), the p85 regulatory subunit of phosphoinositide 3'-kinase (PI3'K) and other adapter proteins such as Crk and Nck [23]. Thus, enzymatic activation of the IGF-I-R tyrosine kinase results in stimulation of an array of various intracellular signaling cascades, including the Ras/Raf/MAP kinase and PI3'-kinase pathways. Other signaling pathways are also involved in IGF-I-R-mediated biological outcomes. Many of the protein kinase C (PKC) isoforms are regulated by IGF-I, and in vascular smooth muscle cells, for example, downregulation of these isoforms results in inhibition of IGF-I-stimulated DNA synthesis and cell migration. PKC may also activate the Ras/Raf/MAP kinase pathway. Adapter proteins, including the Crk family of protooncogenes, are involved in the IGF-

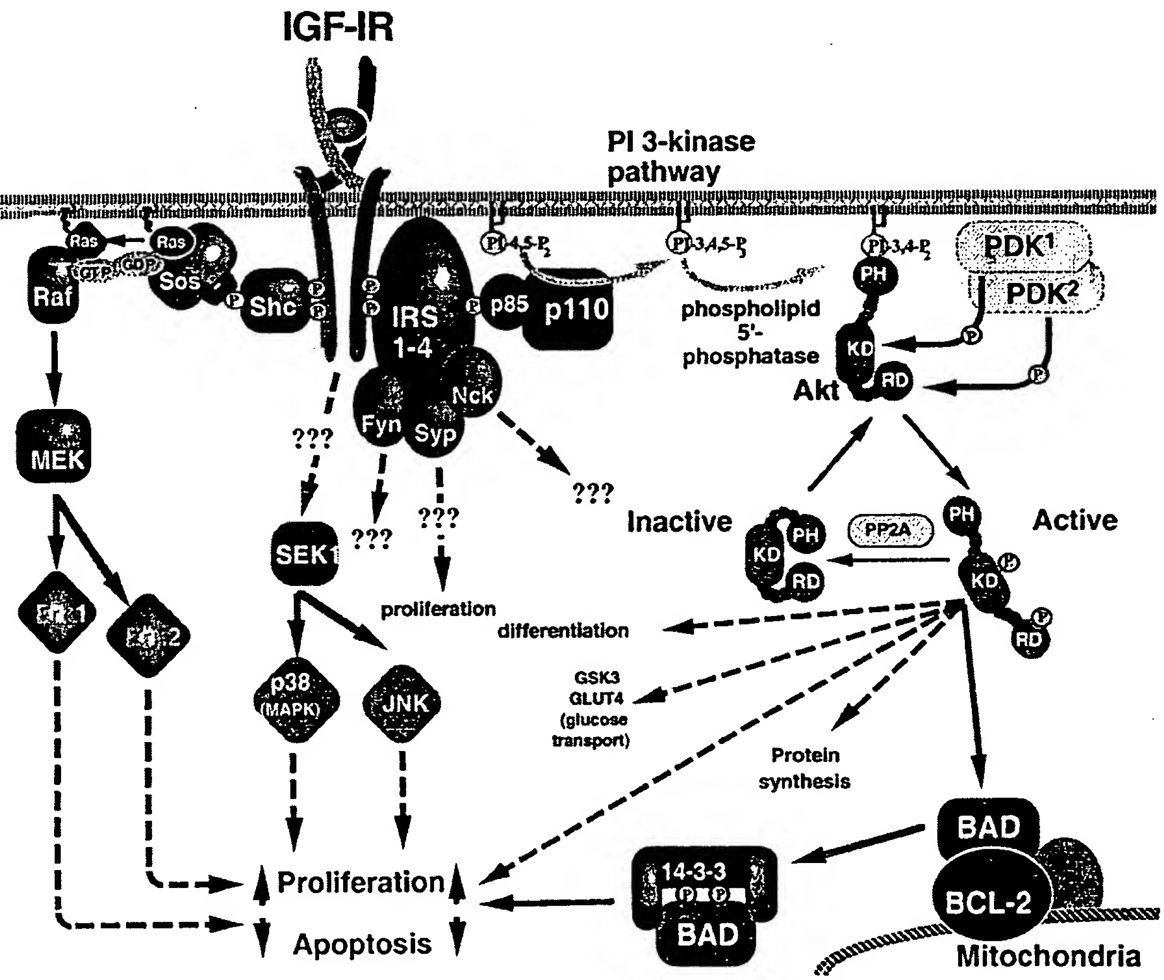

Figure 1. Schematic representation of intracellular signaling pathways emanating from the IGF-I receptor. The activated IGF-I receptor phosphorylates a number of adaptor proteins, including IRS-1, IRS-2 and SHC. These tyrosine phosphoproteins then interact with downstream molecules, e.g. p85, Syp, Grb2 and Nck, via their SH2 domains, thereby activating the Ras/Raf/MAP kinase, the PI3'-kinase and other kinase pathways.

Table 1. Characteristics of IGF-system knockout mice.

Disrupted gene	Birth weight (% normal)	Perinatal viability	Characteristics
IGF-I*·†	60	5–70	genetic background affects perinatal viability, delayed ossification, underdeveloped muscles and lungs, infertility
IGF-II‡	60	100	normal postnatal (but not catchup) growth, develop into fertile dwarfs
IGF-I-R*	45	0	severe in utero growth retardation, hypoplasia, abnormal skin formation, delayed bone development, abnormal CNS
IGF-II/Man-6-P§	125–140	0	moderate fetal overgrowth, cardiac hypertrophy

* [29].
† [28].
‡ [27].
§ [34].

I-R signaling cascade, and CrkL expression, for example, results in a transformed phenotype [24, 25].
Activation of the IGF-I-R both increases mitogenesis and inhibits apoptosis. Until recently, IGF-induced mitogenesis was attributed primarily to the Ras/Raf/MAP kinase pathway, whereas the antiapoptotic effects of IGFs were thought to be mediated by the PI3′-kinase pathway [1, 11]. IGF-I-R activation has been shown to inhibit apoptosis and induce phosphorylation of downstream substrates such as AKT/PKB and Bad in a PI3′-kinase-dependent manner. Phosphorylation of Bad causes it to dissociate from Bcl2, thereby allowing Bcl2 to inhibit apoptosis. Whereas the PI3′-kinase pathway may be important in many cell lines for IGF-I-R inhibition of apoptosis, other pathways, including the MAP kinase pathway, have also been implicated in this effect of IGF-I. Still other pathways and substrates that have been implicated in IGF-I-R signaling include Grb10 and the Ca^{2+}/cyclic AMP response element-binding protein (CREB). Indeed, activation of CREB as well as the MAP kinase pathways leads to regulation of the expression of a large number of genes [1, 21, 22].
In the past, IGF-I-R signaling pathways (like growth factor receptor signaling cascades in general) have been studied and presented as linear tracts. However, it has become increasingly clear over the past few years that many if not all of these pathways interact with each other and that there is significant cross-talk between tyrosine kinase receptors and other cell surface receptors such as G-protein-related serpentine receptors, cytokine receptors and integrins. Thus, synthetic $\alpha V\beta 3$ integrin receptor antagonists inhibit IGF-I-stimulated smooth muscle cell migration and replication, β-arrestins can regulate IGF-I-R-induced mitogenesis, and antiestrogens affect the expression and phosphorylation state of the IGF-I-R, to mention just a few examples [11].

IGF knockout models

Homologous recombination has recently been used to selectively disrupt the expression of genes encoding ligands and receptors of the IGF family (table 1). This approach has proven invaluable to assess the specific roles of these proteins in embryonic and postnatal growth [26–29]. Thus, targeted disruption of the IGF-II gene resulted in mice that weigh just 60% that of their normal littermates at the time of birth. However, this reduction in growth rate was restricted to the embryonic period, and the animals developed into essentially normal and fertile dwarfs. The phenotype of null mutants for the IGF-I gene is apparently more complex, and seems to depend on the genetic background of the animals. Thus, some of these mice died shortly after birth, whereas others survived and reached adulthood. Surviving IGF-I-null mice showed, among other abnormalities, a delay in the ossification process, underdeveloped muscles and lungs, and infertility. Mice heterozygous for the disrupted IGF-I gene exhibited no major growth retardation. The phenotype of mice with a liver-specific disruption of the IGF-I gene and the implications of these observations on the somatomedin hypothesis will be described in the next section.
Homozygous IGF-I-R knockout mice exhibited the most severe developmental retardation. These animals, which invariably died at birth, weighed only 45% that of normal controls. They displayed hypoplasia, abnormal skin formation, delayed bone development and anomalous central nervous system morphology. Given the widespread distribution of the IGF-I-R during ontogenesis, it seems that this receptor can mediate the endocrine effects of IGF-II, which circulates in both the bloodstream and in cerebrospinal fluid, as well as the autocrine/paracrine actions of locally produced IGFs [30, 31]. The extensive damage resulting from disruption of the IGF-I-R gene is therefore consistent with its central role as a cell survival factor [32]. In contrast,

ablation of the IR gene resulted in mice that weighed ~ 90% of normal weight and showed no major developmental delay at time of birth. These animals, however, died during the first several days of postnatal life as a result of diabetic ketoacidosis [33]. Thus, these homologous receptors have clearly distinct functional roles in vivo.

Finally, disruption of the IGF-II/Man-6-P receptor resulted in mice that were 25–40% larger than nomal at birth [34]. In addition, these animals displayed cardiac hypertrophy and died shortly after birth. The excessive growth in these animals could potentially be explained by the increased levels of circulating IGF-II (up to ~ 4.5-fold above normal values), which are likely to be a compensatory response to the absence of a functional IGF-II/Man-6-P receptor. Consistent with this interpretation, double knockouts of IGF-II and the IGF-II/Man-6-P receptor are rescued from perinatal lethality [35].

In humans, loss of one copy of the IGF-I-R gene has been reported in a number of infants with deletion of the distal long arm of chromosome 15 (q26.1 → qter) [36, 37]. An additional genetic rearrangement, ring chromosome 15, has been also documented to result in the loss of one allele of the IGF-I-R gene [38]. Most patients showing deletion of the distal 15q portion, as well as those with the ring chromosome, have severe intrauterine and postnatal growth retardation. Homozygous partial deletion of the IGF-I gene has been reported in a 15-year-old boy with severe prenatal and postnatal growth deficiency, sensorineural deafness and mental retardation [39]. This phenotype suggests that there is an important role for IGF-I not only in postnatal but also in prenatal growth. Moreover, the observed neurological deficiencies clearly point to a crucial role for IGF-I in normal development of the central nervous system [39].

Liver-specific IGF-I knockout: implications on the somatomedin theory

The original somatomedin hypothesis suggested that GH regulated the production of somatomedin (later called IGF-I) and that this factor was produced primarily by the liver, and reached peripheral target tissues via the circulation. Furthermore, the somatomedin hypothesis suggested that IGF-I was the major mediator of GH action. Subsequent studies demonstrated that IGF-I was produced by all tissues and probably has local autocrine/paracrine effects ('modified somatomedin hypothesis'), though distinguishing between the endocrine and the autocrine/paracrine roles of IGF-I has been elusive. Modern technology now makes it possible to address these questions by conditionally knocking out specific genes in specific tissues.

To create a liver-specific deletion of the IGF-I gene, we generated transgenic mice expressing Cre recombinase exclusively in the liver by expressing Cre under the control of the albumin promoter [6]. Cross-breeding of the loxP-flanked IGF-I mice and the albumin-Cre-expressing mice resulted in deletion of the IGF-I gene in the liver. IGF-I mRNA levels in liver were < 1% of the levels in wild-type animals. In contrast, IGF-I mRNA levels measured in nonhepatic tissues such as heart, muscle, fat, spleen and kidney were similar to those of control animals.

Circulating levels of IGF-I in these animals were markedly reduced (25% of those in wild-type animals) at 6 weeks of age. Postnatal growth and development, as assessed from age 3 to 6 weeks, was normal. Sexual maturation was normal, as demonstrated by normal fertility, normal-size litters and normal lactation and weaning. There were essentially no phenotypic distinctions between the liver-specific IGF-I-gene-deleted animals and their wild-type littermates. Thus, although liver production of IGF-I is the major contributor to circulating 'endocrine' IGF-I levels (~ 75%), liver production of IGF-I is not essential for normal postnatal and pubertal growth and development in the mouse. Rather, autocrine/paracrine IGF-I production is sufficient for normal growth and development. Thus, both the original and modified somatomedin hypotheses need to be reevaluated.

The IGFs as potential predictors of breast and prostate cancer

The role played by the IGF system in the biology of human cancer has generated a great deal of attention from both basic and clinical researchers [40, 41]. IGF-I functions as a progression factor during the cell cycle: once the cell is stimulated to enter G1 by a competence factor such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) or other stimuli, the cell will be able to traverse the cell cycle solely in the presence of subphysiologic concentrations of IGF-I [42]. In fact, most primary tumors and transformed cell lines express high levels of IGF-II mRNA and protein, with some tumors overexpressing the IGF-I gene [40]. IGF-I and IGF-II are assumed to act in an autocrine manner via the IGF-I-R.

The recent finding that higher circulating IGF-I concentrations (and lower IGFBP-3 levels) are associated with an increased risk of breast and prostate cancer sparked an ardent debate on the issue of whether endocrine levels of IGF-I can be used as predictors of specific types of cancer [43, 44] (table 2). The first of these prospective studies included 121,700 women (aged 30–55 years), with 397 cases of breast cancer confirmed

among individuals in which the levels of IGF-I and IGFBP-3 were measured on average 28 months before diagnosis. The relative risk (RR) of breast cancer in premenopausal women less than 50 years old was 4.6 in the upper tertile of IGF-I values (compared with individuals in the lower tertile). When the concentrations of IGFBP-3 were incorporated into the multivariate analysis, the RR increased to 7.3. The second prospective study included 15,000 men (aged 40–82 years), with 152 cases of prostate cancer confirmed (on average after 7 years) in individuals in which the concentrations of IGF-I and IGFBP-3 were determined. The RR of prostate cancer in the upper quartile of IGF-I values was 2.4 compared with the lower quartile, although it increased to 4.3 when the concentrations of IGFBP-3 were included in the analysis. In men over 60 years of age the RR was 7.9.

The results of these investigations, if confirmed by additional large prospective studies, may shed light on the role of circulating IGF ligands and binding proteins on the etiology of a variety of human cancers. Furthermore, such studies may potentially facilitate the design of more rational hormonal therapies intended to lower IGF concentrations and/or to decrease the sensitivity of the target organs to the mitogenic action of the IGFs.

The IGF-I receptor and cancer

A fundamental role of the IGF-I-R in malignant transformation has now been well established. Several clinical and experimental observations support this notion: (i) the IGF-I-R is highly overexpressed by most tumors and cancer cell lines [40, 45]; (ii) fibroblast cell lines (R^-) established from mouse embryos in which the IGF-I-R was disrupted by homologous recombination cannot be transformed by any of a number of oncogenes, including the SV40 large T antigen, activated *ras* and the bovine papillomavirus E5 protein [46–48]. Reintroduction of a functional receptor renders R^- cells susceptible to the transforming activities of these oncogenes; and (iii) overexpression of the IGF-I-R results in a ligand-dependent transformed phenotype which includes the formation of tumors in nude mice [49].

The transforming activity of the IGF-I-R depends, to a large extent, on its potent antiapoptotic activity, in addition to its mitogenic effects. The ability of the IGF-I-R to protect cells from apoptosis has been shown in several different systems, including fibroblasts, neurally derived cells, hemopoietic cells and others [50, 51]. Especially impressive was the antiapoptotic activity displayed by the IGF-I-R in vivo [52, 53]. These experiments showed that R^- cells undergo apoptosis when placed in a biodiffusion chamber in the subcutaneous tissue of a rat. In contrast, fibroblasts overexpressing the receptor, or tumor-derived cells with elevated numbers of receptors, had the capacity to double over a 1-day period. Furthermore, the major single factor determining cell survival in these studies proved to be the number of IGF-I-Rs [52]. IGF-I-R levels have been shown to be the critical determinant that causes cells to switch from a 'nonmitogenic' to a 'mitogenic' mode. Thus, one study demonstrated that cells with less than 15,000 IGF-I-Rs will not grow in serum-free media supplemented with IGF-I, whereas cells with 22,000 binding sites will grow in the sole presence of IGF-I. Furthermore, cells expressing more than 30,000 receptors are able to grow in soft agar, suggesting an increase in their transforming capacity.

In light of the central role played by this receptor in many transforming events, targeting the IGF-I-R as a potential anticancer therapy appears to be a promising approach. Potential strategies include the use of anti-receptor antibodies, ligand analogs and antisense methodologies. Induction of apoptosis appears to be the common theme of these different modalities. In view of the potential relevance of the endocrine IGF-I and IGFBP levels on cancer predisposition, it may be that logical and comprehensive therapeutic approaches designed to simultaneously target ligands, receptors and binding proteins may have the greatest probability of success [54].

Table 2. Correlation between circulating levels of IGF-I and risk of breast* and prostate† cancer.

Plasma IGF-I	RR	RR‡
Breast cancer (premenopausal, <50 years old)		
<158 ng/ml	1.0	1.0
158–206 ng/ml	2.64	3.12
>207 ng/ml	4.58	7.28
RR = relative risk		
Prostate cancer		
99–184 ng/ml	1.0	1.0
185–236 ng/ml	1.32	1.94
237–293 ng/ml	1.81	2.83
294–500 ng/ml	2.41	4.32

* [44].
† [43].
‡ Adjusted for IGFBP-3.

Interplay between oncogenes and tumor suppressors in control of IGF-I receptor gene expression

Some of the transcription factors that regulate expression of the IGF-I-R gene have now been identified. Characterization of the mechanisms of action of these factors has provided important information that is facil-

itating our understanding of the molecular events responsible for IGF-I-R expression in normal and pathologic states. Expression of the IGF-I-R gene is regulated by both positive and negative factors. The first group comprises a number of growth factors and oncogenic agents that positively affect cell division (mitogenic agents), whereas the second group includes negative modulators of cell growth such as tumor suppressors.

Growth factors that have been shown to stimulate transcription of the IGF-I-R gene include basic FGF and PDGF [55–57]. bFGF, for example, has been shown to increase receptor binding and mRNA levels, and this effect has been mapped to a region of the proximal IGF-I-R promoter localized between nucleotides − 476 and − 188 in the 5′-flanking region of the gene. Likewise, PDGF increased the activity of the IGF-I-R promoter via an ~100-bp promoter fragment located immediately upstream of the transcription start site. Since this region has a canonical c-*myc* binding site, and since PDGF induces c-*myc*, the effect of PDGF on IGF-I-R expression may be mediated by c-*myc*. Interestingly, upregulation of the IGF-I-R by bFGF and PDGF is consistent with the hypothesis that the main role of these competence factors is to generate enough IGF-I and IGF-I-R to induce the growth response [58, 59]. In contrast, expression of the IGF-I-R gene is negatively regulated by the local concentrations of IGF-I [55, 60].

IGF-I-R gene expression is induced by steroid hormones in addition to peptide growth factors. Treatment of MCF-7 cells and normal breast xenografts with estradiol increased IGF-I-R mRNA levels two- to threefold, whereas progesterone treatment decreased these levels by ~50% [61]. These results indicate that augmenting the concentration of the IGF-I-R, and thereby increasing the responsiveness of the organ to the circulating or locally produced IGFs, is a potential mechanism by which estradiol stimulates cellular proliferation.

The IGF-I-R promoter is also targeted by multiple oncogenes. Constitutive overexpression of the protooncogene c-*myb* in Balb/c-3T3 cells has been shown to abrogate the requirement for IGF-I in the growing media. This effect of c-*myb* was associated with an increase in the levels of both IGF-I and IGF-I-R mRNAs [62, 63]. Another oncogene known to stimulate IGF-I-R promoter activity is the hepatitis B virus X (HBx) protein. In hepatocellular carcinoma-derived cell lines containing HBx protein, endogenous levels of the IGF-I-R mRNA were increased approximately fivefold compared with controls [64]. The implication of these findings is that HBx may play a role in the etiology of hepatocellular carcinoma by stimulating the expression of the IGF-I-R gene.

In addition to controlling transcription of the IGF-I-R gene, oncogenes can also affect IGF-I-R action by nontranscriptional mechanisms. For instance, transformation of human cells by $pp60^{src}$, the product of the *src* oncogene of the Rous sarcoma virus, results in constitutive phosphorylation of the receptor β-subunit, whereas addition of IGF-I further increases the level of phosphorylation. $pp60^{src}$ thus induces the ligand-independent phosphorylation and activation of the IGF-I-R, thereby subjecting the cell to a constitutively mitogenic signal [65, 66].

Tumor suppressors, a family of negative growth regulators, have been linked to the development of a wide variety of human cancers, including breast, colon and lung cancer [67–69]. Due to the central role of the IGF-I-R in cell cycle progression and transformation, it has been postulated that a potential mechanism by which the postmitotic, fully differentiated cell is kept out of the cell cycle may involve the constitutive inhibition of the IGF-I-R gene by wild-type tumor suppressors [70].

WT1 is a tumor suppressor whose inactivation has been linked to the pathogenesis of a subset of Wilms' tumors, a pediatric kidney neoplasia [71]. WT1, via its zinc-finger DNA binding domain, has been shown to bind to specific cis elements in the IGF-I-R promoter region, and to suppress the activity of transfected promoter fragments as well as the endogenous levels of IGF-I-R mRNA [72–74]. Loss of WT1 activity in Wilms' tumor and related malignancies (resulting from chromosomal deletions, missense or nonsense mutations, translocations or alternative splicing) may result in transcriptional derepression of the IGF-I-R gene. Activation of the overexpressed receptor by circulating or locally produced IGFs may be a key step in the biology of Wilms' tumor.

A particular case in which WT1 has been shown to be disrupted is the case of the desmoplastic small, round cell tumor (DSRCT), a very aggressive abdominal tumor. DSRCT is characterized by a recurrent translocation [t(11;22)(p13;q12)] that joins the N-terminal (activation) domain of EWS1 (the ubiquitously expressed Ewings' sarcoma gene) to the C-terminal (DNA binding) domain of WT1 [75–77]. Pathologic fusion of EWS to WT1 has been shown to abrogate the tumor suppressor function of WT1 and to generate an oncogenic chimeric protein capable of binding and activating the IGF-I-R promoter [78]. This gain-of-function event constitutes a novel paradigm in oncogenesis.

Likewise, p53, which is the most frequently mutated tumor suppressor, is capable of suppressing the activity of the IGF-I-R promoter, as well as endogenous levels of IGF-I-R mRNA. In contrast, tumor-derived, mutant versions of p53 significantly stimulated promoter activity [79, 80]. It is reasonable to speculate that part of the

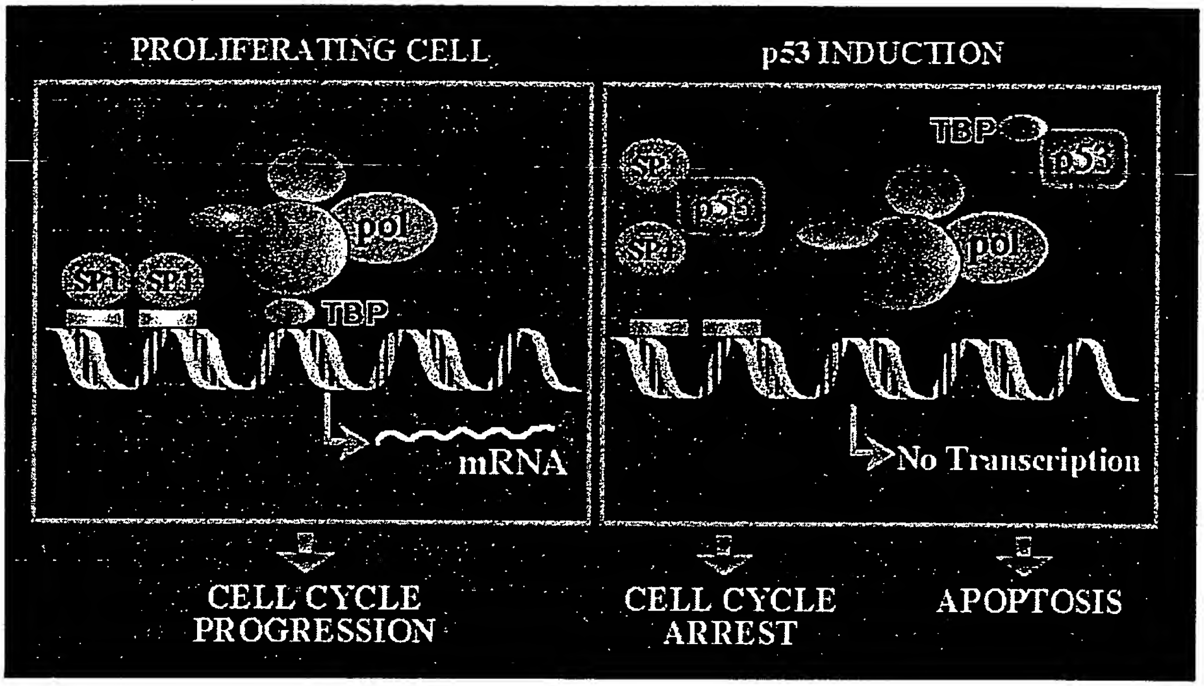

Figure 2. Model for p53 suppression of IGF-I receptor promoter. The IGF-I-R promoter contains multiple binding sites for transcription factor SP1, a zinc-finger nuclear protein that strongly stimulates transcription from a specific group of RNA-polymerase II-dependent promoters in vertebrates. In addition, transcription from the IGF-I-R gene seems to depend on the binding of TBP (the TATA-binding protein) to the 'initiator' region, with ensuing assembling of a functional transcription initiation complex that includes RNA polymerase-II (pol). p53, which is usually induced following cellular insults such as DNA damage, can bind both SP1 and TBP, thus precluding these proteins from binding specifically to the IGF-I-R promoter region. Consequently, transcription from the IGF-I-R gene is impaired, and as a result, cell cycle arrest or apoptosis may occur.

effects of p53 on cell cycle arrest and apoptosis are mediated via suppression of the IGF-I-R promoter [fig. 2]. This may therefore imply that lack of suppression by mutant p53 in tumors may facilitate expansion of a malignant population of cells. Importantly, additional components of the IGF system are regulated by p53. That is, transcription of the IGF-II gene is similarly reduced by wild-type p53 [81], whereas IGFBP3 (which usually functions as an inhibitor of IGF action) is stimulated by p53 [82]. In conclusion, p53 controls the IGF signaling system by regulating expression of ligands, receptors and binding proteins.

Interactions between wild-type and mutant forms of these transcription factors are very complicated and may involve additional DNA-binding and non-DNA-binding interacting proteins. It is likely that a finely tuned interplay between these stimulatory and inhibitory factors ultimately determines the level of expression of the IGF-I-R gene and the proliferative status of the cell. A clear understanding of these interactions will prove important in our attempts to target the IGF-I-R as a potential therapeutic approach

Future directions

The IGF system of ligands, receptors and binding proteins is undoubtedly a major player in normal cellular growth and differentiation. These elements also play important roles in aberrant growth as seen in neoplastic disorders. Whereas the IGFs and the IGF-I-R have not been shown to be, by themselves, oncogenic, evidence has evolved that strongly suggest that they may enhance proliferation of preneoplastic and neoplastic cells. Attacking this ubiquitous system of growth factors for adjunct therapies in cancer patients is therefore an obvious new and hopefully fruitful direction of research.

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Exhibit C

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COMMENTARY

Insulin-Like Growth Factors and Skeletal Growth: Possibilities for Therapeutic Interventions

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In this, her last issue as editor-in-chief of JCEM, Dr. Maria New has invited some of her colleagues in pediatric endocrinology to contribute short commentaries on issues of current interest. It is a pleasure to honor Dr. New on this occasion, both for her leadership in taking our journal to a new level of excellence and for her many scientific contributions that have enhanced our understanding and treatment of adrenal disorders.

The central focus of pediatric endocrinology has been the hormonal control of growth and development, and in recent decades it has been possible to correlate the serum concentrations of nearly every known hormone with each phase of normal growth and with most types of aberrant growth. The insulin-like growth factors (IGFs) or somatomedins, as they are frequently called, have been an important focus of pediatric endocrinologists because of their pivotal role in skeletal growth. The present essay addresses the roles of these peptides in the growth of the skeleton and considers how we might take advantage of their many actions for therapeutic purposes.

Overview of IGF ligands, receptors, and biological actions

The two known somatomedins, IGF-I and IGF-II, are structurally homologous to proinsulin. They were initially identified on the basis of three unique properties: their mediation of the skeletal growth-promoting actions of GH, their mitogenic properties, and their mimicry of the actions of insulin. These peptides were isolated in Zurich by Rinderknecht and Humbel on the basis of their insulin-like activity, but were renamed IGF-I and IGF-II when it became apparent that their growth-promoting properties were more important than their insulin-like activities (1). Somatomedin C was isolated in Chapel Hill from human blood fractions by Van Wyk *et al.* guided by assays based on the stimulation of proteoglycan and DNA synthesis in cartilage as suggested by William Daughaday. Somatomedin C was found on sequence analysis to be identical to IGF-I as reported by the Zurich group (2). As no peptide comparable to IGF-II was isolated under the somatomedin rubric, it is now conventional to use IGF-I and IGF-II when referring to the specific peptides and the term somatomedins or IGFs when referring to these peptides generically (3).

The IGFs are more primitive hormones than insulin and have a much broader spectrum of action. Insulin presumably evolved from the IGFs to fulfill a more specialized function when the need arose to store energy during periods of fasting. The ancestral relationship of insulin to IGFs to insulin is similar to that of PTH to PTH-related protein (PTHrP), with PTHrP having a more ubiquitous spectrum of activities than PTH.

Both of the somatomedins are developmentally regulated and subject to precise tissue-specific expression. Methods for the quantitation of IGF-I were developed before comparable methods became available for IGF-II (4, 5), and to this day much less is known about the physiological roles of IGF-II than those of IGF-I. Blood levels of IGF-II are considerably higher than those of IGF-I, and the concentrations of IGF-II in extracts of skeletal tissue are higher than those of IGF-I. Blood levels of IGF-I are more stringently regulated by GH and nutritional factors than are those of IGF-II, but IGF-II is more insulin like than IGF-I. The idea has been perpetuated that IGF-II is the fetal hormone and IGF-I is the somatomedin of postnatal life. It is now clear, however, that both IGF-I and IGF-II are important for normal fetal growth and that both have distinctive functions postnatally.

Most of the IGF-I circulating in blood comes from the liver, where the expression of the IGF-I gene is regulated by GH (6). The IGFs are also produced in peripheral tissues, where the expression of their genes is regulated by many hormones. IGFs are required for the proliferation of most cell types, and they promote cell survival by inhibiting programmed cell death (7). They also regulate a vast number of highly differentiated cell functions.

The nature of the responses to IGFs is dependent on physiological circumstance. It is pertinent to the role of IGFs in skeletal growth that sparsely plated chondrocytes respond to IGF-I with increased DNA synthesis, but not with increased sulfate incorporation into proteoglycans. On the other hand,

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when the same cells reach confluence, IGF-I stimulates matrix synthesis but has no effect on DNA synthesis. Such observations imply that IGFs may act as both a mitogen in the proliferative zone and a stimulant of proteoglycan synthesis in the hypertropic zones of the growth plate, with the specific effect being dependant on the cellular and hormonal environments.

The biological importance of the somatomedins is often underestimated because their effects, compared with those of other growth factors, are often weak or difficult to demonstrate. This is because the IGFs characteristically exercise a permissive or modulating role on biological processes rather than serving as the primary agonist. This was illustrated in the interactions of IGF-I with pituitary hormones in the gonads and thyroid. Adashi *et al.* showed in rat granulosa cells that the effect of FSH plus IGF-I was nearly 10-fold greater than the effect of FSH by itself, even though IGF-I by itself was seemingly inactive (8). Similarly, whereas IGF-I is a relatively weak mitogen in rat thyroid cells, its mitogenicity is potentiated more than 30-fold after these cells are exposed to TSH (9).

Both IGF-I and IGF-II produce their biological effects through type I receptors that are homologous with the insulin receptor (10). Insulin and the IGFs all cross-react with the type I and insulin receptors and with hybrid receptors containing subunits of each receptor. Type I receptors are present in most, if not all, tissues. The cytoplasmic domain of the type I receptors contains a tyrosine kinase that initiates a phosphorylation cascade through the Ras-Raf-mitogen-activated protein kinase and phosphoinositol 3'-kinase pathways (7). A variety of docking proteins, including insulin receptor substrate-1 and -2 and Shc, act as immediate substrates for the receptor tyrosine kinase. Type I receptors have been observed in proliferating chondrocytes from several species, with predominant expression in developing chondrocytes (11, 12). The IGF-II/mannose-6-PO_4 receptor protects against toxic levels of IGF-II, but is not believed to mediate the actions of IGF.

IGF-binding proteins (IGFBPs)

Secretion of IGFBPs accompanies the secretion of IGFs in most, if not all, tissues in which IGFs are made. The patterns of IGFBP secretion vary considerably between different tissues and in response to differing physiological circumstances, thus imparting a high degree of specificity to the actions of the IGFs. The specific endoproteases that degrade the several IGFBPs are likewise hormonally regulated and provide additional mechanisms for regulating IGF actions.

Each of the six major IGFBPs has been identified in skeletal tissues. In osteoblast-like cells, IGFBP-3, -4, and -5 are probably the most important based on their relative abundance and biological potencies (13). The addition of exogenous IGFBPs to bone cells has produced both stimulatory and inhibitory effects on IGF actions (14). IGFBP-4 does not bind to cells, and it inhibits bone cell proliferation by competing with the IGF receptor for binding the IGFs (15). By contrast, IGFBP-3 and -5 enhance receptor binding and the anabolic effect of the IGFs on bone (16).

IGFBPs may be responsible for the high levels of IGF-I and IGF-II in bone matrix. IGFBP-3 binds to proteoglycans prepared from a variety of cartilage tissues, and the bound IGFBP-3 sequesters IGF-I in extracellular matrix (17). Matrix proteoglycans do not bind IGFs in the absence of IGFBP-3. IGFBP-5 has strong affinity for hydroxyapatite, and in the absence of IGFBP-5, the IGFs do not bind to hydroxyapatite (18).

The importance of binding proteins as modulators of IGF action suggests that the most promising possibilities for exploiting IGFs as therapeutic agents in skeletal disease may depend on finding ways to selectively manipulate IGFBPs. This might be accomplished by altering their expression or proteolytic degradation or by discovering substances that compete with IGFs for binding to the binding proteins. Another approach has been to modify IGF-I by truncating the amino-terminus. Such peptides [*e.g.* des(1–3)-IGF-I] have markedly reduced affinity for binding proteins and in many systems are far more active than native IGFs (19).

Role of IGFs in skeletal growth

An early issue was whether GH acts directly on skeletal tissues to stimulate growth, or whether its growth-promoting actions require the mediation of IGF-I as stipulated in the somatomedin hypothesis of GH action. Although it is now well established that IGFs are essential for normal skeletal growth, arguments have persisted concerning whether the IGF-I that mediates skeletal growth is derived from the peripheral circulation or is synthesized in the growth plate in response to GH and other hormones that stimulate its synthesis locally (20). In our opinion this is not a substantive issue, because both endocrine and paracrine modalities have been well documented *in vivo*. Targeted knockout of the IGF-I gene in the liver (but not in other tissues) by the Cre/loxP system does not impair the growth-promoting actions of GH, even though blood levels of IGF-I are substantially decreased (21).

Controversies also remain, however, over whether IGF-I by itself can stimulate the proliferation of chondrocytes or can do so only after GH has first stimulated the differentiation of prechondrocytes into more mature cartilage cells, as postulated by Green's dual effector theory of GH action (22). The ability of IGF-I to restore growth in dwarfed children who lack functional GH receptors suggests that IGF-I delivered from the circulation is sufficient to produce growth without the need for GH (23). Such responses, however, attenuate over time and are less than those observed with GH treatment of GH deficiency states. Ohlsson has postulated that GH is required for providing an adequate stem cell population of prechondrocytes (24).

Evidence from gene deletion studies on the role of IGFs in skeletal growth

Definition of the roles of the somatomedins in skeletal growth has been obtained from gene knockout models in mice lacking the genes encoding IGF-I, IGF-II, or their respective receptors. Knockout studies confirmed that both IGF-I and IGF-II are essential for normal prenatal growth, as mice deficient in either IGF-I or IGF-II were only 60% of normal size at birth (25). Knockout of IGF-II did not alter

postnatal growth, as the size ratio between normal and mutant littermates was maintained into adulthood. Unlike the IGF-II-deficient mice, most of the IGF-I mutant mice died at birth, and those that survived showed severe postnatal growth retardation.

Mice with gene knockout of the type I IGF receptor had even greater fetal growth deficits (45% of normal birth weight) than knockouts of either IGF-I or IGF-II alone (26, 27). Mice lacking the type I IGF receptor exhibited delayed appearance of their ossification centers and delay in epiphyseal maturation, and they usually died at birth due to generalized hypoplasia of all muscles, including those responsible for respiration. Experiments with combined deletions made clear that the type I receptor mediates the essential functions for IGF-I and most of those for IGF-II, at least during fetal development.

Gene knockout studies revealed that a primary function of the IGF-II receptor (IGF-II/M6Pr) is to regulate IGF-II levels, which can be lethal if too high (28). A null mutation in the IGF-II/M6Pr gene resulted in large birth weights and lethality of nearly all mutant embryos. Simultaneous deletion of the IGF-II gene completely rescued the phenotype associated with the IGF-II/M6Pr gene knockout, because high levels of IGF-II were avoided (29).

Genetic models of selective GH or IGF-I deficiency in humans confirm the rodent knockouts. A child reported by Woods *et al.*, who had an inactivating mutation of the IGF-I gene, suffered severe intrauterine and postnatal growth retardation (30). In contrast, children who lack the GH gene or who are unresponsive to GH are essentially normal at birth, and their growth retardation is limited to postnatal life. From this we can conclude that IGF-I and IGF-II, but not GH, are essential for normal intrauterine skeletal growth.

Immortalized T lymphocytes from a tribe of African Pygmies have a decreased number of type I IGF receptors, and those present are not phosphorylated and do not transmit a signal in response to IGF-I (31). Baserga and colleagues have shown that the proliferation of transgenic cells lacking the IGF type I receptor is markedly impaired, and that they do not respond to epidermal growth factor and other mitogens (32).

Synthesis and biological actions of IGFs in skeletal tissue

Skeletal tissues are rich in IGFs. Baylink *et al.* found that extracts from demineralized human femora removed during hip replacement procedures contain a factor(s) that stimulates cell division in rat calvaria. The active ingredient that they provisionally called skeletal growth factor was subsequently purified and found on sequence analysis to be IGF-II (33).

The formation of IGFs, their receptors, and their binding proteins in skeletal tissues is regulated by many hormones, including GH, estradiol, testosterone, bone morphogenic proteins, PTH, PTHrP, 1,25-dihydroxyvitamin D_3, and a variety of cytokines and growth factors. At least some of the actions of sex steroids on bone are dependent on their stimulation of IGF production. Estradiol stimulation of cell proliferation and collagen synthesis in rat osteoblasts can be blocked by anti-IGF-I antibodies (34), and testosterone-dependant growth of rat mandibular condyles is also blocked by an antibody to IGF-I (12). In female reproductive tissues, not only is the IGF-I gene responsive to estrogen (35), but, conversely, IGF-I can activate the estrogen receptor in the absence of estrogen (36). There is also cross-talk between the IGF and estrogen signal transduction pathways (37). Such interactions may provide an important key to manipulating IGFs for therapeutic advantage.

The location of IGF production in the epiphyses is pertinent to their role in skeletal growth. Induction of IGF-I messenger ribonucleic acid by GH has been demonstrated in the rodent growth plate, and IGF-I transcripts have been localized to both proliferating and hypertropic chondrocytes. *In situ* studies, however, suggest minimal expression of IGF-I messenger ribonucleic acid in rodent epiphyses and high expression of the gene encoding IGF-II (11, 38).

It is intriguing to speculate on how abnormal epiphyseal maturation in certain chondrodysplasia might be influenced by the interactions of IGF-I with PTHrP or with fibroblast growth factor (FGF; Fig. 1). PTHrP (or PTH) stimulates chondrocyte proliferation at least in part by stimulating increased production of IGF-I; indeed, PTH stimulation of osteoclasts is blocked by an anti-IGF-I antibody (39). The commitment of chondrocytes to further differentiation, however, is inhibited by PTHrP, an action that is opposite that of the IGFs (40). Knockouts of the PTH/PTHrP receptor display accelerated chondrocyte differentiation and disordered organization. Blömstrand chondrodysplasia, a form of short-limbed dwarfism, and Jansen's metaphyseal chondrodysplasia are both caused by mutated PTHrP receptors in the chondrocyte (41, 42). Thus, a potentially fruitful area for further research is to determine how IGFs influence the effect of PTHrP on chondrocyte differentiation under normal conditions and in the chondrodysplasias resulting from disordered expression of the PTH/PTHrP receptor.

Other forms of chondrodystrophies, including achondroplasia, hypochondroplasia, and thanatophoric dysplasia, are caused by gain of function mutations of the FGF receptor (43) that act to decrease chondrocyte proliferation and cellular hypertrophy (44). Studies in growth plate chondrocytes from a fetus with thanotropic dwarfism suggest that the receptor mutation led to premature terminal differentiation and increased apoptosis of chondrocytes in response to FGF (45). The enhanced apoptosis is again opposite the effects of IGF-I. IGF-I inhibits apoptosis in osteoblasts, and survival of these cells is blocked by an IGF-I receptor antibody. It remains to be demonstrated whether the antiapoptotic effects of IGF-I can block the effects of the mutant FGF receptor gene in these hereditary chondrodystrophies (46).

Interactions among IGFs, GH, and sex steroids in pubertal growth

The evidence that IGF-I plays a pivotal role in pubertal growth is substantial. In both sexes blood levels of both GH and IGF-I rise dramatically during the second decade, implicating the GH/IGF axis in the pubertal growth spurt (47), and in both sexes, estrogen is the primary stimulus for increased GH secretion and the consequent rise in circulating IGF-I (48). In boys this is presumably accomplished by aromatization of testosterone to estradiol, as nonaromatizable androgens do not exert a GH-priming effect.

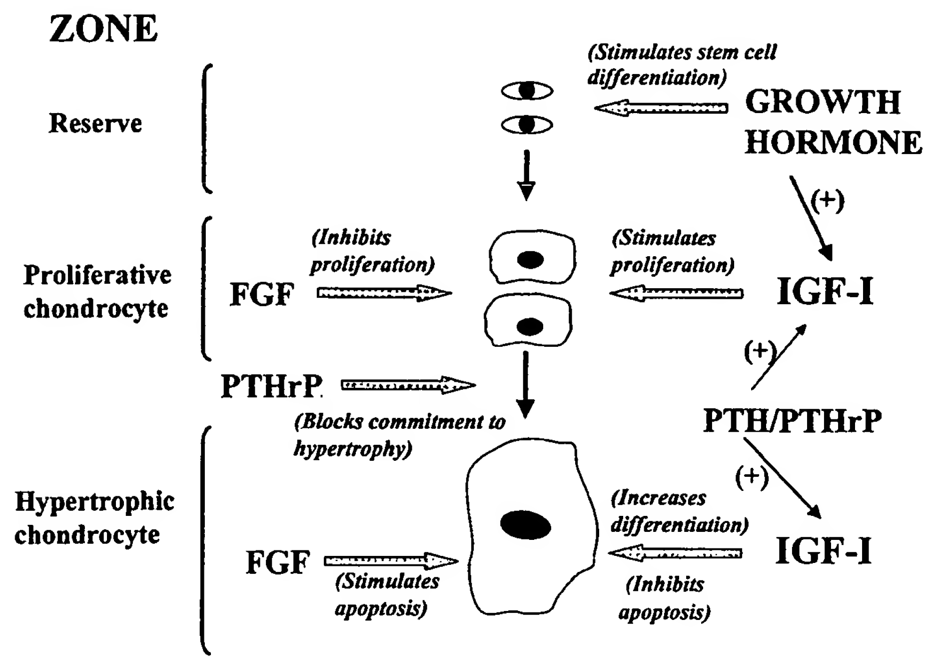

FIG. 1. Many of the chondrodystrophies are the result of mutant genes encoding the receptors for FGF or PTHrP. This figure illustrates that the actions of FGF and PTHrP on the growth plate in these diseases are opposite the actions of IGFs, suggesting possible therapeutic approaches to circumvent the growth impairment in these disorders.

There is poor correlation between circulating levels of sex steroids and IGF levels at puberty, possibly due to direct actions of sex steroids on the growth plate. Androgen receptors (49) and both α and β estrogen receptors (50) have been localized to human growth plate chondrocytes, and dihydrotestosterone and estradiol have direct actions on human chondrocytes. Another factor contributing to the poor correlation between circulating levels of estradiol and IGF-I may be the biphasic action of estrogen on growth and GH/IGF expression. Low or physiological doses of estrogen augment statural growth (51), whereas the high doses of estrogen used to treat acromegaly or tall stature in girls suppress IGF-I levels and reduce acral and statural growth (52).

New insights on the roles of sex steroids and IGFs in pubertal growth have come from the identification of three rare patients whose epiphyses failed to fuse as the result of disrupted estrogen action. Smith *et al.* reported a tall, 28-yr-old, normally masculinized man whose epiphyses had not yet fused and who was unresponsive to large doses of exogenous estrogen. His lack of bone maturation was secondary to a loss of function mutation of estrogen receptor α (53). Two men with p450 aromatase deficiency have been described with a similar phenotype (54, 55). Failure of aromatization caused profound estrogen deficiency and produced skeletal lesions similar to those in the patient with estrogen insensitivity. Treatment of the latter two patients with exogenous estrogens led to epiphyseal fusion and improvement in their skeletal lesions. In all three of these patients the IGF-I levels were appropriate for skeletal age.

These experiments of nature show that estrogen is responsible in both males and females for the terminal phases of epiphyseal differentiation leading to fusion, and that IGF-I is unable to effect epiphyseal fusion in the absence of estrogen. These men, however, were well virilized and attained their midparental height at an appropriate age. Although it is tempting to attribute their estrogen-independent growth during childhood to their normal androgen levels, it is equally possible that their normal growth was attributable to IGF-I. The latter possibility is supported by the relatively normal statural growth of individuals with primary hypogonadism and an intact GH/IGF axis (56). The ability of IGF-I to promote linear growth without unduly accelerating skeletal maturation has important therapeutic implications.

Potential therapeutic usages and frontiers for further study

The skeletal disorders that could theoretically be benefited from augmenting or decreasing the actions of IGF-I or IGF-II include various forms of arthritis, glucocorticoid-induced growth arrest, renal osteodystrophies, various chondrodystrophies, healing of fractures, the prevention and treatment of osteoporosis, and many others. Many of the potential uses of IGFs in skeletal disorders have been inferred from therapeutic trials with GH, even though the effects of GH may be quite different from those of IGF-I. GH has a host of direct effects that are catabolic and antiinsulin in nature. These effects are opposite the indirect anabolic effects of GH that are mediated by IGF-I (57). For example, the growth failure that is an invariable feature of high dosage glucocorticoid therapy may be amenable to IGF-I therapy with less chance of glucose intolerance than might be expected from treatment with GH.

The most obvious clinical use for IGF-I is to treat children who are unable to respond to GH itself. The growth responses to IGF-I in these children have been impressive, although not as sustained as those in GH-deficient children treated with GH itself. Underwood *et al.*, who have treated 10 such children for longer than 5 yr, have encountered no serious side-effects, although the children regularly have developed mild enlargement of abdominal viscera and lym-

phoid tissue (23). Similar findings have not been observed in children treated with GH.

Except in primary IGF-I-deficient states, there are significant disadvantages to the systemic administration of IGF-I or IGF-II for organ-specific purposes. IGF receptors are present in virtually every cell in the body, and exposure to pharmacological concentrations of exogenously administered IGFs are likely to produce unpredictable and unwanted side-effects. Enthusiasm for treating insulin-dependent diabetes, for example, waned when concerns arose that IGF-I might be exacerbating diabetic retinopathy.

Despite the ubiquity of the IGFs and their receptors, the body is able to selectively manipulate the actions of the IGFs by taking advantage of the many layers of regulation that govern their actions. The challenge, therefore, is not only to increase our understanding of the roles that IGFs play under varying pathological circumstances, but to learn how to manipulate these effects in a selective and beneficial manner.

The most obvious approach is to modify access of the appropriate ligand to the target tissue. Because IGF-II is more abundant than IGF-I in blood and skeletal tissue, future studies would profit from comparing the effects of IGF-I and IGF-II with one another and with those of modified forms that do not interact with the IGFBPs.

The six major binding proteins and the proteolytic enzymes that degrade them offer nearly inexhaustible opportunities for selectively modulating the availability of biologically active IGFs to specific targets. A recent example of this approach was a study in transgenic mice in which the smooth muscle hyperplasia induced by selectively overexpressing IGF-I was partially abrogated by concomitantly overexpressing the inhibitory IGFBP-4 (58). Binding proteins might also be manipulated through the hormones that regulate their production and/or degradation or by using modified IGFs that do not attach to binding proteins.

A different approach to capitalizing on the many actions of IGFs in skeletal tissue is to modify the effects of other hormones whose actions are mediated or impacted by IGF. An example is the use of estrogen antagonists that selectively block the effects of estrogen on epiphyseal fusion while retaining their beneficial effects on bone mass. Case reports suggest that tamoxifen, while promoting bone mineralization, may be an estrogen antagonist at the level of the chondrocyte maturation. This is supported by anecdotal reports that tamoxifen treatment can lead to normal statural growth without stimulating bone age advancement in patients with elevated estrogens secondary to McCune-Albright syndrome. In an experimental study, the accelerating effect of estrogen on bone age advancement in mice was blocked by Faslodex, an estrogen receptor blocker (59).

Gene therapy may ultimately prove to be a viable technique for achieving the goals outlined above. Although the enormous potential of gene therapy has proven far more difficult to realize than originally envisioned, it is likely that in coming years we will gain expertise in targeting designer genes to fulfill specific objectives at discrete anatomical sites. To successfully target a modified gene to a specific area or function in the growth plate, it will be important to identify unique promoters or cell-specific fingerprints for the multiple chondrocyte stages in growing bone. Realization of the enormous potential of targeted gene therapy to treat skeletal disorders, however, requires far more knowledge than we now possess on how IGFs function in collaboration with other hormones and growth factors to influence each stage of skeletal growth.

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